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DIARRHEA DIGEST

DIARRHEA DIGEST is an irregular publication of TECHLAB® dedicated to the etiology, diagnosis, and therapy of diarrheal diseases and related aspects of intestinal ecology

SPRING 2006

C. difficile … a new and " improved" version of an old pathogen?

… reported by Katie Couric, host of The Today Show

… featured in Science News, the Weekly News Magazine of Science in an article entitled "Flora Horror: hospitals struggle with a serious new gut microbe"

… "a new and emerging pathogen" by Dr. Clifford McDonald at the CDC

… quoting Dr. Sandra Dial at McGill University, "Ten years ago, we didn't believe people died of this. It was very unusual. Now, unfortunately, it's not unusual."

… the topic of symposia at CDC, Digestive Disease Week, and ICAAC 2006

The subject of these reports is *C. difficile*, a gram positive anaerobe that causes antibiotic associated diarrhea and colitis. Are these reports a lot of false hype? Unfortunately, no. Healthcare professionals know the name *C. difficile*, and up until several years ago, most believed that we had control over this anaerobe. Now there are more outbreaks than ever. In fact the disease is being seen in persons that we used to think were not susceptible --- outpatients and even young healthy adults who were not being treated with antibiotics and who apparently had healthy GI tracts.

The outbreaks are significant and most seem to be associated with a particular isolate designated NAP1/027. In Canada, outbreaks due to this strain have resulted in the death of more than a thousand patients in a single 12-month period. This is more than 5 times the normal mortality rate. The predisposing condition, by far, continues to be treatment with antibiotics, but the more we learn about *C. difficile*, the more challenging this pathogen seems to be.

What is C. difficile NAP1? NAP1, more accurately designated as NAP1/027, is responsible for many of the severe *C. difficile* outbreaks now being reported. The designation NAP1/027 stands for toxinotype III, North American pulsed field gel electrophoresis type 1, PCR ribotype 027.

The dogma has been that all you have to do to treat this "antibiotic-associated" disease is give the patient another antibiotic, either metronidazole or vancomycin. Fair enough. As a result, *C. difficile* has moved down the priority list in terms of "important pathogens". Simply put, the ability of this organism to "out-maneuver" us was overlooked. After all, just because other pathogens had become more virulent didn't mean that it would happen with *C. difficile*.

Unfortunately, it has happened. Now that *C. difficile* has leapfrogged over our attempts to control it, we should try to look carefully at the primary outbreak isolate and determine whether it truly is different from the more classical clinical isolates of the 1980's and 90's.

Twenty-five years ago, when we first began building our collection of *C. difficile* isolates, we had quite a few that produced high levels of toxin. Some were from the collection of Dr. John Bartlett, one of the pioneers in *C. difficile* research. Some were from the VPI anaerobe collection and others were from clinical collections in Europe. Many of the basic properties that have been established for *C. difficile* were determined with VPI strain 10463,

WARNING! This newsletter contains explicit intestinal information and portions may be rated BS for content. Readers are advised to proceed at their own discretion.

a strain that produces very high levels of toxins A and B. Only by using strains such as these were researchers able to purify enough toxin for the basic biological studies on this pathogen and its disease. Many published articles involved toxin from weakly toxigenic strains, leading to inaccurate characterization of the toxins.

If we fast-forward to 2005-2006 to the new outbreak strain, we see reports that this strain produces very high levels of toxins A and B. This high level expression is reportedly due to lack of toxin regulation by *tcd*C, a small open reading frame that is part of the pathogenicity islet of *C. difficile*. However, the control of toxins A and B expression by *tcd*C is still poorly understood, and additional research is needed to more accurately demonstrate its control over the toxins (more accurately, over the *tcd*A and *tcd*B genes). Even so, there has been speculation that the outbreaks and increased severity are due to increased toxin production. A point that is overlooked in most of these reports, however, is the fact that the outbreak strain produces less toxin than many of the high toxin producers characterized more than 25 years ago. So increased toxin production alone does not seem to be the only factor involved in increased virulence.

What else is novel about NAP1/027? How about its ability to produce a binary toxin (also called an iota toxin) --- a so-called third toxin. A binary toxin is a toxin comprised of two non-linked protein components. In this case, there is an enzymatic component and a binding component. Both must be present to be toxic.

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TEL: In the U.S., 1-800-TechLab
Outside the U.S., 540-953-1664

FAX: 540-953-1665

Email: techlab@techlab.com

Website: www.techlab.com

Comments or suggestions about the *DIARRHEA DIGEST* are greatly appreciated. If you would like to be added to or deleted from the mailing list, please notify us.

Types of toxigenic C. difficile strains (so far)

- Toxin A+, toxin B+
- Toxin A+, toxin B+, binary toxin+
- Toxin A-, variant toxin B+
- Toxin A+, variant toxin B+, binary toxin+

NOTE: Variant toxin B is more cytotoxic and lethal than toxin B; binary toxin is also referred to as iota toxin or CDT

Either one alone is not toxic. Several years ago, strains that carried both components began to be identified. We can say with certainty that the outbreak strain carries the genes for the binary toxin. Most clinical isolates of *C. difficile* do not carry these genes, and where the genes come from is anyone's guess. Other clostridia, in particular *C. perfringens* and *C. spiroforme*, produce binary toxin (more appropriately referred to as iota toxin with these two species) that has been shown to be a virulence factor for both of these species and the only virulence factor for *C. spiroforme*.

The binary toxin of *C. difficile* is highly related, but not identical to binary toxin from its relatives. Ancestrally speaking, there likely was a primordial gene that gave rise to the binary toxin, but when it diverged and how the outbreak isolate picked up its version is good stuff for a graduate student seminar. Is the binary toxin a new virulence factor for *C. difficile*? At this time, all we can say is that it does not appear to be highly active, and how well it is expressed is not clear. However, the presence of this toxin certainly complicates the pathogenic profile of *C. difficile* (see inset).

Then there is the factor that, in our opinion, possibly represents the greatest threat. The outbreak isolate is resistant to fluoroquinolones. At first, we viewed this with some degree of skepticism. After all, we and others in the field knew that *C. difficile* was certainly susceptible to commonly used antibiotics. At the same time, there were many healthcare professionals who we felt incorrectly believed that the disease occurred because *C. difficile* was indeed resistant to antibiotics. The science showed, however, that *C. difficile* was sensitive to antibiotics, and that it mainly got its foothold in the colon as the antibiotic therapy ended. Once in the colon, it was able to grow and

compete with the "re-establishing" flora, resulting in infection and disease.

Now, to our dismay, it seems that the outbreak strain truly is resistant to fluoroquinolones (we have seen this with our own eyes) --- and we can now agree with healthcare professionals who correctly believe that the disease occurs because *C. difficile* is resistant to fluoroquinolones. In other words, *C. difficile* does not have to wait until the antibiotic levels drop before it can begin to grow. It can infect the patient and start growing in the colon while the patient is on fluoroquinolones. This ability should be very unsettling to all of us. Fluoroquinolones are one of the most frequently prescribed antibiotics in hospitals, and patients who are being treated with them are going to be highly susceptible. Their normal flora will be wiped out, and their colons will be "ripe" for infection.

In summary, what can we say about the outbreak isolate? We can say that the isolate has properties that give it the potential to be hypervirulent --- it produces high amounts of toxin, although not as much as some early clinical isolates. It also carries a new toxin, but no one knows yet what this new toxin contributes in the way of disease. And very importantly, this isolate is resistant to fluoroquinolones, a property that makes it especially a threat in patients being treated with this class of antibiotics. But this is not the end of the story. We have no doubt that there will continue to be new discoveries about this pathogen that the healthcare system (including us as *C. difficile* researchers), at one time, thought it understood. *C. difficile* may not be a new pathogen, but we certainly are dealing with a more dangerous version.

Do current in vitro diagnostic tests detect the C. difficile outbreak strain NAP1/027? All of our findings show that NAP1/027 produces toxins A and B and common antigen (glutamate dehydrogenase) that react in all of our *C. difficile* tests. As far as we can tell, they should work in other commercial tests that are specific for the toxins or for antigen. Therefore, these tests will be effective *in vitro* diagnostic aids for patients infected with NAP1/027, just as with other strains. To determine if you have the outbreak strain at your facility, other tests (e.g., bacterial isolation, toxinotyping, and pulsed field gel electrophoresis) are required.

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C. difficile and Inflammatory bowel disease …

In just-released abstracts that will be featured at Digestive Disease Week and the 107th Annual Meeting of the American Gastroenterological Association Institute, there is new information on the increased incidence of *C. difficile* disease in patients with inflammatory bowel disease (IBD). In our own studies, we have demonstrated *C. difficile* and its toxins in patients with ulcerative colitis and occasionally in Crohn's disease patients, but the real challenge for the physician is trying to determine which set of events is responsible for the symptoms. Are the symptoms caused by a *C. difficile* infection, or is this actually a flare of an existing IBD condition? Both involve inflammatory processes. Even in the absence of pseudomembranes, most cases of *C. difficile* disease are accompanied by inflammation due to the tissue damage

caused by the toxins and their own chemotactic properties.

A new test for measuring anti-Saccharomyces cerevisiae antibodies (ASCA) as a diagnostic aid for Crohn's Disease

The detection of anti-*Saccharomyces cerevisiae* antibodies (ASCA) in serum as an indicator of Crohn's disease is a common diagnostic tool for assessing subjects suspected of having inflammatory bowel disease (IBD). Even though these antibodies are only present in approximately 50 to 60% of adult Crohn's patients, the specificity is high enough to offer a clinical utility for differentiating Crohn's disease from ulcerative colitis and from other gastrointestinal illnesses like irritable bowel syndrome (IBS). In pediatric IBD, the sensitivity of serum ASCA drops to about 45% while maintaining a similar specificity as seen in adult IBD. In the healthy population, ASCA is present in 5 to 6% of subjects while showing a slight increase to 10 to 15% in subjects with ulcerative colitis. No one really knows exactly why there is an antibody response to *Saccharomyces cerevisiae*, commonly known as baker's yeast, a common part of our everyday diets. Currently, there is no evidence to show that the yeast is involved with the disease itself. The primary IgG and IgA antibody responses are to the yeast mannan of the cell wall. The simplest explanation for now is that there is homology between the yeast mannan and an unidentified self-antigen that is part of the autoimmune response in Crohn's disease. Even though ASCA is present in both active and inactive Crohn' disease, studies have shown that titers increase during flares of disease. The presence of higher ASCA titers in Crohn's disease has been correlated to more severe disease and decreased periods between complications leading to surgery. These observations have fueled a second potential diagnostic use for monitoring disease activity in Crohn's disease using titers of ASCA.

More recently, a newly patented approach has been introduced for the measurement of ASCA in feces. The same antibodies that are detected in serum can be measured in a fecal specimen as an aid in the diagnosis of Crohn's disease. The newly cleared TECHLAB *ASCA-CHEK*® test is an ELISA that is optimized for the detection of human ASCA in feces. The combination of immobilized yeast antigens and a polyvalent anti-human immunoglobulin (Ig) conjugate that captures both IgA and IgG allow for a sensitive and specific noninvasive assay. The test procedure is simple and includes a 1:10 sample dilution with results available in less than 2 hours. When human ASCA is present in the fecal specimen, the specific antibodies bind to the *Saccharomyces cerevisiae* antigens that are immobilized in the test well. Following this step, the polyvalent anti-human horseradish peroxidase (HRP) conjugate binds to the ASCA and reacts with the substrate to produce a positive result. The presence of fecal ASCA is an indicator of Crohn's disease within the setting of differentiating Crohn's disease from ulcerative colitis. This noninvasive diagnostic method is simple to perform and offers the advantage of utilizing fecal specimens for the analysis.

In a clinical study involving 4 separate healthcare sites that included both adult and pediatric subjects, a total of 353 subjects suffering with gastrointestinal illnesses were assessed for disease type and activity and for fecal ASCA. The male to female ratio was approximately 1:1 that is similar to the ratio typically observed in IBD patient populations. The ages ranged from 3 to 78 years. Of the total IBD group, 92 (51.1%) were ≤ 18 years. Results showed an overall sensitivity of 57% and a specificity of 91% for indicating Crohn's disease. When results were stratified by age, the sensitivity decreased slightly to 48% while maintaining a specificity of 92% for indicating pediatric Crohn's disease. In a group of adult IBD subjects, the presence of fecal ASCA was correlated with more severe disease that required surgery ($p=0.02$). This same observation has been made with serum ASCA. In both pediatric and adult Crohn's disease, detectable levels of fecal ASCA are maintained over time during periods of remission and flare. In an earlier clinical study, a female subject suffering with Crohn's disease was monitored for fecal ASCA over a 4-month period using the *ASCA-CHEK*® test. A single fecal specimen was collected at sampling points ranging from day 1 to day 122 and tested by the *ASCA-CHEK*® test. A total of 5

fecal specimens remained *ASCA-CHEK*® test positive during the 4-month period. The OD_{450} results ranged from 0.211 to 0.722 with a mean ± SD of 0.500 ± 0.212 and 95% Confidence Interval of 0.237 to 0.763.

In a separate clinical evaluation, paired fecal and serum specimens were collected from 47 Crohn's disease, 23 ulcerative colitis and 12 non-IBD patients and healthy controls. The test population included both pediatric and adult subjects. All of the fecal specimens were tested using the *ASCA-CHEK*® test and serum specimens were analyzed using the QUANTA Lite™ IgG test (Inova Diagnostics, San Diego, CA). The overall agreement between both tests was 79%. Discrepant results between both tests included fecal ASCA-positive only Crohn's disease and serum ASCA-positive only Crohn's. Results suggest that testing fecal and serum specimens may be the most sensitive method for measuring ASCA. Further studies are needed to determine the combined sensitivity for fecal and serum ASCA.

In times of increasing healthcare costs rapid noninvasive diagnostics are playing a larger role in medical assessments. The new TechLab *ASCA-CHEK*® test offers a rapid and inexpensive method for measuring ASCA as an aid in diagnosing Crohn's disease for both adult and pediatric patients. A significant advantage of this new assay is that testing can be coupled with diagnostic tests for fecal lactoferrin, a marker of intestinal inflammation, including the *IBD-SCAN*® (quantitative) and the *IBD-CHEK*® (qualitative) as a diagnostic panel for assessing patients with chronic gastrointestinal illnesses.

For information on the ASCA-CHEK® test (Cat. #T5016), please contact TECHLAB, Inc.

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Biological paper mills

Which of the following animals meets this description --- large and kind of hairy, makes paper, and has four knees. Is it (a) horse, (b) elk, (c) kangaroo, (d) elephant, or (e) an ex-professional wrestler who's had two knee replacements. If you guessed either the elephant or the wrestler, you would be correct if the ex-wrestler works at a papermill. If he doesn't, then the only correct answer is (d) elephant. The elephant is the only animal listed above with four knees (check the anatomy books for yourself). As for making paper, any of the first four answers --- horse, elk, kangaroo, or elephant --- would be correct.

Each of these animals is what is referred to as a "non-ruminant" animal. They don't thoroughly digest their food, and as a result, their dung contains high levels of plant fibers, which are very good for making high quality paper. The elephant is particularly adept at not digesting its meals. As much as 60% of an elephant's meal goes undigested, and their dung is loaded with plant fiber. Elephants defecate hundreds of pounds each day, and their dung is a ready source of starting material for making paper (100 lbs will give you about 400 good sized sheets of paper). The dung from a healthy elephant has very little smell. If it has much smell, then the elephant probably is sick. The dung is collected, bleached, boiled, and spun. At this point, it is rolled into balls about the size of oranges. Whenever paper is made, each ball is mixed with water, and the mixture is flattened out in framed screens and dried into paper sheets in the sun (this gives new meaning to "three sheets to the wind").

The color and texture of the paper varies, depending on the individual elephant and its diet. If the dung is from an older elephant, the paper is more coarse since older elephants don't chew their food as thoroughly as a younger elephant. The diet affects the coloration --- no surprise there! If you want whiter paper, then you feed the

elephant something like coconuts. For darker paper, you feed the elephant palm branches. Boxes, stationary, notebooks, photo papers, business cards --- all are available from elephant dung. The business cards are said to be "quite the conversation piece" because the first thing people do is smell it once they've been told it's made of dung. Personally, we would have guessed that "dropping the card" would be the first reaction. And as for envelopes … we hope they are self-adhesive. As for manufacturing and marketing costs, dung paper sells for around 10¢ a sheet.

There's talk of making toilet paper from dung, which at first glance seems appropriate. But if you are considering elephant dung as the starting source, then there's the texture to keep in mind --- remember that paper prepared from older elephants which don't chew their food well may have odd bits such as seeds --- so perhaps the process with elephant dung needs to be more "refined" before it can hope to replace Charmin. The seeds may make its use as toilet paper a little less desirable, but actually may make the paper more artsy and increase its value! There is a source --- marsupial manure --- that has been used for toilet paper, and a company called Creative Paper Tasmania, has made the first batch. The paper is sand-colored, and has the notation "Genuine Kangaroo Poo". No report yet, on its performance but the company hopes to drive home its message that the paper is environmentally friendly.

Are these dung products really being used? Of course they are. The business is growing, and companies in other countries are giving it a whirl. In Australia, there is dung paper from kangaroos, and in Scandinavia, there is elk dung paper. It seems likely, however, that the companies in Africa and Sri Lanka will grow faster because they have a better source of starting material --- elephants. By the way, when President Bush and his entourage visited Sri Lanka in 2002, he and others were given writing paper, envelopes, and name cards made on gold-monogrammed elephant dung paper as gifts. Let's see … elephants … Republican party … recycling … hmmm … there ought to be a campaign slogan in here somewhere, if we can just get the dung out of it.

For more information on this subject, check out www.elephantdungpaper.com. The website is great and you'll be amazed at what you'll learn. Even the fact that one of the pioneers in the dung paper business used his wife's kitchen blender to purée the dung mixture prior to making the paper. Perhaps the aromatic flavor of their evening meals raised her suspicions!

Speaking of elephants …

Did you hear the one about the lady who spotted an elephant in her vegetable garden? This was the first elephant she had ever seen, and the woman couldn't tell the front end from the back end. The poor woman was hysterical as she called the police to report "a huge beast in her cabbage patch."

"Exactly what is this beast doing?" asked the policeman.

"The beast is pulling up my cabbages with its tail …" exclaimed the woman.

"And what is the beast doing with the cabbages?" asked the policeman.

"You wouldn't believe me if I told you!" declared the woman.

Can you imagine giving an enema to a cat? Check out "I gave my cat an enema" starring Fred the cat at http://www.catenema.com/cat1.html. The last photo says it all.

Where does it all go and what are we going to do with it?

We humans take a lot of things for granted. One of those is the toilet --- which by the way, we consider to be one of the greatest inventions of the human mind! When we flush the toilet, our "waste" just disappears and we don't worry about it any more. In this country, we are producing tens of millions of tons of the stuff each year, so

it must go somewhere. But because it just disappears, we don't worry about it. That is, unless the toilet gets stopped up, in which case, we know where it's going if we don't fix the toilet. But usually we don't spend much time thinking about it.

But there's an even bigger problem with "waste", and it's based on the fact that it really is difficult to imagine just how much of this stuff there is in this country ---literally. In the late 1990's (we're unsure of more recent figures but it's probably increased), 1.4 billion tons of manure was produced by the animals in the meat industry --- cows and pigs and chickens --- each year. This is at least a hundred times more than the U.S. population produces. This number doesn't include what is produced by us, our pets, or wildlife --- or even what the dust mites in our pillows and bed linens produce --- which according to recent news, is a whole heck of a lot! These meat industry animals are producing a lot of manure, making it even more imperative to find out where this stuff really goes.

A lot of it ends up as runoff into streams, rivers, ponds, and lakes. If you happen to hike and camp, and see a nice clear running stream, chances are the water carries off a lot of the waste as runoff into larger water sources. In most countries around the world, much of the fecal waste ends up as fertilizer. Pig manure, for example, that ends up in large fecal cesspool lagoons on some of the large hog farms often is "injected" into the soil as fertilizer. Chicken manure, and there's plenty of this to go around, has to age a little before it can be used as fertilizer. Otherwise, it is too toxic. Once aged, it becomes a good fertilizer, but are we actually going to "store" the stuff while it ages? After all, this isn't wine we're talking about.

Cow manure is the most abundant, and much of this often is spread on fields as fertilizer. However, many fields become "over-fertilized". The manure is a great source of nitrogen and other minerals, but too much manure can result in too much phosphorus in the soil. This isn't good. It's unhealthy for the environment and unhealthy for us because it eventually contaminates our water sources, and decreases the quality of the water. There are guidelines for the amount of manure that should be used to fertilize fields, but these often are not followed.

Simply put, in addition to getting rid of our own waste, we have too many animals for the amount of land that is being fertilized and we cannot get rid of all the waste. Many environmental companies are trying to develop methods that utilize the methane in manure as an energy source. Although this sounds great, the efficiency of the current processes needs to improve. There also is the problem with leftover biomass, and toxic gases such as hydrogen sulfide that are emitted during the process. The problem of too much manure is a real challenge because it is a real threat to our environment. The question of "Where does it go?" is rapidly becoming one of "Where will it go?" It's time for the human mind to conjure up another great invention!

Upcoming Meetings

Anaerobe 2006, Boise, Idaho, July 25-28, 2006

46th Annual ICAAC, San Franciso, CA, September 27-30, 2006

American Society of Tropical Medicine and Hygiene, Atlanta, GA, November 12-16, 2006

Clostpath 2006. 5th International Meeting on the Molecular Biology and Pathogenesis of Clostridia. Nottingham, England. June 21-25, 2006

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2001 Kraft Drive
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